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Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity

A monolayer study

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Monomolecular layers of lipids at the air/water interface have been used as a model membrane to study membrane interactions of the lantibiotic nisin. The natural lantibiotics nisin A and nisin Z proved to have a high affinity for the anionic lipids phosphatidylglycerol and bis(phosphatidyl)glycerol (cardiolipin). The interaction with zwitterionic phopholipids or neutral lipids is very low at surface pressures higher than 32 mN/m. Nisin, nisin mutants and lacticin 481 show a remarkable correlation between antimicrobial activity and anionic lipid interaction. The results indicate that primarily the N-terminal part (residues 1–22) penetrates into the lipid phase. Reduction of the flexibility at positions 20–21 has a negative effect on monolayer interaction and activity. The C-terminal part is probably responsible for ionic interactions of nisin in monomeric or oligomeric form with anionic lipids. In mixtures of anionic and zwitterionic lipids maximal interactions are found at approximately 70 mol/100 mol anionic lipid. Gram-positive bacteria, which form the main target for nisin, are characterized by a high content of anionic lipids in the membrane. Monolayers formed of lipid extracts of bacteria sensitive to nisin were more strongly penetrated than those of bacteria relatively insensitive to nisin.

Keywords: lipid monolayers; nisin; nisin mutant peptides; anionic lipids; antimicrobial activity.

The antimicrobial peptide nisin is secreted by specific strains of *Lactococcus lactis*, and is widely used in food industry as a safe and natural preservative (Delves-Broughton, 1990). Nisin belongs to the rapidly expanding family of lantibiotics (Schnell et al., 1988), a group of antimicrobial peptides characterized by their low molecular mass and by extensive posttranslational modifications (Gross and Morell, 1971; Jung, 1991). Of the 34 amino acid residues in nisin, 13 are modified. These modifications include dehydration of serine and threonine residues, resulting in three dehydroalanine and five dehydrobutyrine residues, respectively. Five of these dehydrated residues subsequently link their Cβ atom to the sulfur atom of five cysteine residues in a specific way, resulting in the characteristic (β-methyl)lanthionine rings. These modification reactions are probably enzyme-catalyzed and likely candidates for these enzymatic reactions have been proposed (Engelke et al., 1992, 1994; Kuipers et al., 1993a; Van der Meer et al., 1993).

Two natural nisin species have been found so far: nisin A, containing a His residue at position 27, and nisin Z, containing an Asn residue at position 27 (Mulders et al., 1991; De Vos et al., 1993; Piard et al., 1992, 1993; Fig. 1). The structural genes for nisin A and Z have been cloned and sequenced (Buchman et al., 1988; Mulders et al., 1991), and both genes are commonly found in nature (De Vos et al., 1993). Both peptides have similar antimicrobial activities, although nisin Z forms larger zones of inhibition in agar-diffusion assays using solid media, probably caused by better diffusion. Another lantibiotic produced by *Lactococcus lactis* is lacticin 481, which has a different antimicrobial spectrum and structure than nisin (Fig. 1).

The organization of the nisin gene cluster *nisABTCIPRKFEG* (Buchman et al. 1988; Kaletta and Entian, 1989; Engelke et al., 1992, 1994; Van der Meer et al., 1993; Kuipers et al., 1993a; Siegers and Entian, 1995), the function of the encoded proteins in biosynthesis (Engelke et al. 1992, 1994; Van der Meer et al., 1993; Kuipers et al., 1993b), nisin precursor secretion (Van der Meer et al., 1993) and immunity (Kuipers et al., 1993a,b; Engelke et al., 1994; Siegers and Entian, 1995), and the way by which the expression of the nisin genes is regulated (Kuipers et al., unpublished results), have been studied in detail. In addition, protein engineering studies of nisin have been performed to gain insight into the requirements for the complicated biosynthesis of nisin and as attempts to improve relevant properties of nisin (Kuipers et al., 1992; Van der Meer et al., 1994; Rollema et al., 1995). By exchanging residues involved in posttranslational modification reactions, one can determine the importance of these residues for antimicrobial activities and for processes such as modification, secretion, immunity and mode of action.

Several studies were directed to unravel the mode of action of nisin, and these have shown that nisin interacts with the cellu-

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Abbreviations. $Ole_2GroPGro$, 1,2-dioleoylglycero-3-phosphoglycerol; $Ole_2GroPCho$, 1,2-dioleoylglycero-3-phosphocholine; $Ole_2GroPEtn$, 1,2-dioleoylglycero-3-phosphoethanolamine; $(Ptd)_2Gro$, bis(phosphatidyl)glycerol, cardiolipin; $Pam_2GroPGro$, 1,2-dipalmitoylglycero-3-phosphoglycerol; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; Abu-S-Ala, β-methyllanthionine; Ala-S-Ala, lanthionine; $acyl_2Gal$-Gro and $acyl_2Gal_2Gro$, diacyl mono- and digalactosyl glycerol. Mutants of nisin are abbreviated according to the Recommendations of Nomenclature Committee [see *Eur. J. Biochem.* (1984) *138*, 35 (Table 6)].

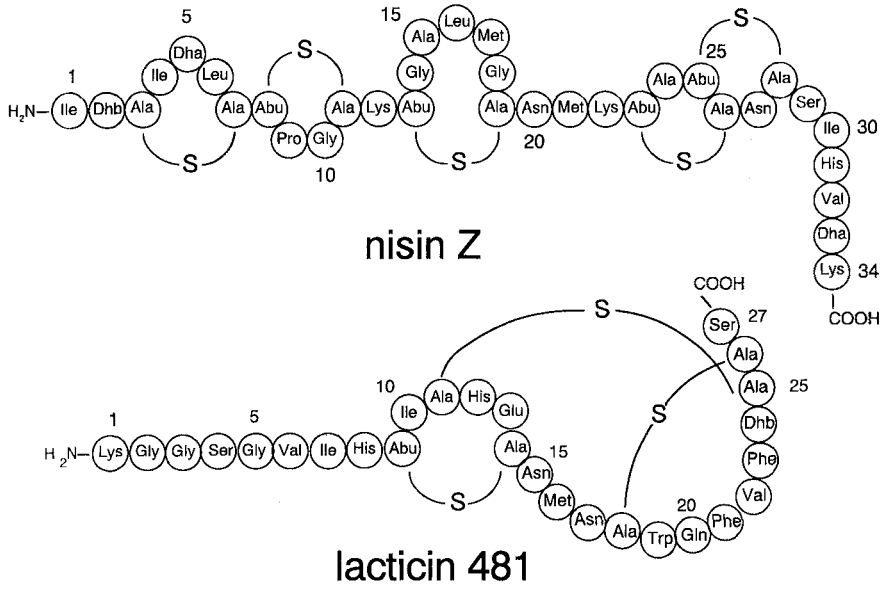

Fig. 1. Primary structure of nisin Z and one of the two possible structures of lacticin 481. The information was taken from Piard et al., 1993.

lar membrane of target organisms, can dissipate the membrane potential by pore formation and eventually kills the cell (Sahl et al., 1987; Sahl 1991; Gao et al., 1991; Driesen et al., 1995). Yet the molecular mechanism is unknown, as well as the importance of specific residues for membrane interaction. For this reason the interactions between wild-type and mutant nisins as well as lacticin 481 (Piard et al., 1993) and phospholipids were studied using monomolecular layers. The monolayer technique has been proven as a valuable tool in the study of membrane-active compounds (Demel, 1994). To gain further insight into the membrane interaction of nisin, we have used lipid monolayers of various composition and monitored the lipid interaction of nisin with the Wilhelmy plate method. This approach allows one to determine lipid specificities and to obtain information on the nature of the interaction of nisin with lipids. In addition, the phospholipid composition of membranes of two target bacteria is determined, and the action of nisin on monolayers of these phospholipids and on monolayers of defined mixtures of phospholipids is studied. A good relationship between *in vivo* activity and phospholipid monolayer penetration of nisin (mutants) was observed.

MATERIALS AND METHODS

Lipids. 1,2-Dioleoylglycero-3-phosphoglycerol (Ole_2GroP-Gro), 1,2-dioleoylglycero-3-phosphocholine (Ole_2GroPCho), 1,2-dioleoylglycero-3-phosphoethanolamine (Ole_2GroPEtn), bis(phosphatidyl)glycerol (cardiolipin, Ptd_2Gro) and phosphatidylinositol (PtdIns) from soybeans were obtained from Avanti Polar Lipids (Birmingham AL). Cardiolipin from *Escherichia coli* (strain AH 930) was isolated and purified as described (Killian et al., 1994). Diacyl monogalactosyl glycerol ($acyl_2GalGro$) and diacyl digalactosyl glycerol ($acyl_2Gal_2Gro$) were isolated from a total pea thylakoid membrane lipid preparation (van't Hof et al., 1991). Dipalmitoylglycerophosphoglycerol (Pam_2-Gro$P$Gro) was synthesized according to established methods (Comfurius and Zwaal, 1977).

Bacterial strains, plasmids, media and growth conditions. *E. coli* strain MC1061 (Casadaban et al., 1980) was used as a recipient strain in cloning experiments. *L. lactis* strains used for expression of mutant nisins were NZ9700 (Kuipers et al., 1993a,b) carrying the nisin−sucrose transposon Tn*5276* (Rauch and De Vos, 1992) and NZ9800, containing a disrupted *nisA*gene on the chromosome. Plasmid vectors used for the cloning experiments were pNZ9013 and pNZ9019 (Kuipers et al., 1992). *E. coli* strains were grown in tryptone/yeast broth at 37°C. *L. lactis* strains were cultivated without aeration at 30°C in M17 broth (Difco Laboratories, Detroit MI), containing 0.5% (mass/vol.) sucrose. For nisin production, cells were grown in SPYS medium (consisting of 1% sucrose, 1% bactopeptone from Difco, 1% yeast extract from Difco, 0.2% NaCl, 0.002% $MgSO_4 \cdot 7 H_2O$ and 1% KH_2PO_4, pH 7.0). For large-scale production, 3% sucrose was added instead of 1%. When appropriate, media were supplemented with 10 μg/ml chloramphenicol both for *E. coli* and *L. lactis*.

DNA manipulations, site-directed mutagenesis and DNA sequence analysis. Plasmid isolations from *E. coli* and transformations of *E. coli* strains were performed according to established procedures (Sambrook et al., 1989). Plasmid DNA from lactococcal cells was isolated and transformed into *L. lactis* by means of electroporation essentially as described previously (Vos et al., 1989). Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from New England Biolabs Inc. (Beverly MA) or Promega Corporation (Madison WI), and used as recommended by the manufacturers. Oligonucleotides used as primers in sequencing and PCR reactions were synthesized in a Cyclone DNA synthesizer (Biosearch, San Rafael CA) or were purchased from Pharmacia. Sequences of the primers used for PCR and site-directed mutagenesis are: 5′-GATTAAATTCTG**C**AGTTTGTTAG-3′ (*Pst*I), 5′-CCCTAAA-**AAG**CTTATAAAAATAGG-3′ (*Hin*dIII) 5′-CAGGTGCATCA-CCACGC**TGG**ACAAGTATTTCGCTATGTAC-3′ (Ile1→Trp), 5′-CACCACGCATT**T**CAAGTATTTCGCTATG-3′ (Dhb2-→Dha), 5′-AACAGGAGCTCTG**TG**GGGTTGTAACATG-3′, (Met17→Trp) and 5′-CTGATGGGTTGT*Δ*AAAACAGCAA-CTTGTCATTG-3′, (des-Asn20, Met21). Mutated nucleotides are indicated in boldface; mutations and restriction sites that were introduced are indicated in parentheses. Site-directed mutagenesis was performed as described before (Kuipers et al., 1991). Sequencing of purified DNA fragments was performed by the dideoxy chain-termination method (Sanger et al., 1977). All mutants were purified to homogeneity and the structures of the modified residues were confirmed by one- and two-dimen-

sional ^{1}H-NMR (Kuipers et al., 1992). It was established that mutant [Dha2]nisin Z contained a Dha residue at position 2, [Thr1]nisin Z and [Thr17]nisin Z contained a Trp residue at position 1 and 17, respectively (unpublished results).

Production, purification and characterization of nisin species. Wild-type nisin Z and its mutants were produced by batch fermentation as described previously (Kuipers et al., 1992). Nisin species were purified by chromatography on Fractogel TSK butyl 650-S followed by reverse-phase HPLC. The latter was performed using a system consisting of a Gilson 231 sample injector, two Waters 510 pumps, a Waters TCM column oven and a Separations model 759A variable-wavelength detector. Waters Maxima 820 software was used for gradient control and data acquisition. Hi-Pore RP318 (Bio-Rad) columns were used: 250×4.6 mm for analytical runs (flow rate 1 ml/min) and 250×21.5 mm for preparative runs (flow rate 10 ml/min). In all experiments the columns were kept at a constant temperature of 30 °C. The elution buffers consisted of 10% aqueous acetonitrile, 0.1% trifluoroacetic acid (buffer A) and 90% aqueous acetonitrile, 0.08% trifluoroacetic acid (buffer B). A typical gradient used in analytical runs was 15–30% B, linear in 60 min. Peak detection was at 220 nm. All nisin species were checked for purity by analytical reverse-phase HPLC and ^{1}H-NMR. ^{1}H-NMR spectra were obtained on a Bruker AM400 spectrometer operating at 400.13 MHz. The samples for NMR contained approximately 3 mM nisin in 10% D_2O/90% H_2O, pH 3.5. All spectra were measured at 25 °C. The solvent resonance was suppressed by prior low-power irradiation. The spectra were referenced to 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)propionic acid. Structures of nisin Z mutants were analyzed by two-dimensional NMR techniques essentially as described previously (Kuipers et al., 1992). Antimicrobial activities of (mutant) nisin Z species were determined as minimal inhibitory concentration values against the indicator strains *Micrococcus flavus* DSM1790, as described before (Kuipers et al., 1992).

Lipid extraction. Lipids were extracted from gram-positive bacteria sensitive to nisin (*Micrococcus flavus*, DSM 1790) and relatively insensitive to nisin (*Listeria monocytogenes*, NIZO collection) by the method of Bligh and Dyer (1959). Cells were harvested in the late exponential phase.

To increase the extractability of anionic lipids, especially bis(phosphatidyl)glycerol, cells were also first treated with lysozyme to degrade glycosidic bonds of the cell-wall glycan layer. A *M. flavus* cell pellet, originating from 2 l cell culture, was suspended in 5 ml 10 mM K_2HPO_4, 0.4 M sucrose, 3 mM EDTA, 1.5 mM EGTA pH 7.8; 42 mg lysozyme (Boehringer, Mannheim) was added and incubated for 20 min at 37 °C (Filqueras and Op den Kamp, 1980). For *L. monocytogenes*, approximately 10 times higher lysozyme concentrations and an additional sonication step before incubation was necessary, to improve the efficiency of cell-wall degradation.

The total lipid fraction was purified on a silica column (Baker particle size 40 µm, pore diameter 6 nm) eluted with chloroform/methanol 1:1 (by vol.). Anionic lipids were converted into the sodium salt (Killian et al., 1994) while the lipid fractions were kept under N_2 at all times to prevent oxidation. Lipid composition was determined by two-dimensional thin-layer chromatography (Silica 60, Fertigplatten, Merck) using chloroform/methanol/ammonia/water (62:28:2:2, by vol.) and chloroform/methanol/acetic acid (65:25:10, by vol.), respectively.

The individual spots were identified using reference lipids and staining with I_2 vapor, phosphorus reagent, Schiff reagent (to indicate phosphatidylglycerol and glycolipids) and orcinol (to indicate glycolipids). Phospholipids were quantified by the method of Rouser et al. (1970).

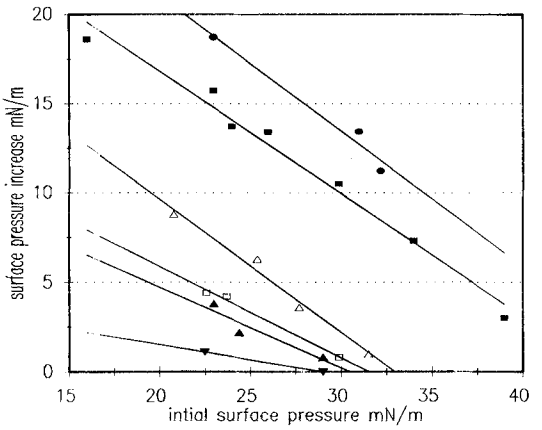

Fig. 2. Surface pressure increases after the injection of nisin Z underneath monolayers of various lipids at different initial surface pressures. *E. coli* Ptd_2Gro (●); $Ole_2GroPGro$ (■); $Ole_2GroPCho$ (△); $acyl_2GalGro$ (□); $Ole_2GroPEtn$ (▲); $acyl_2Gal_2Gro$ (▼). 10 µg nisin Z was injected in a subphase of 5 ml 10 mM Tris pH 7.4.

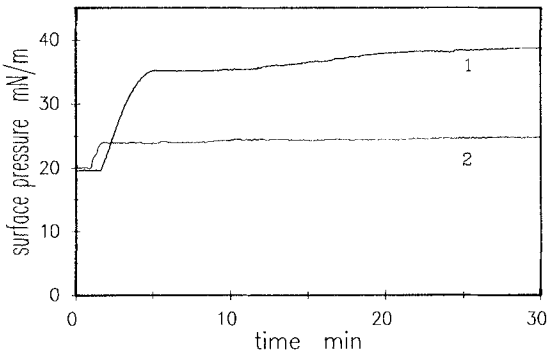

Fig. 3. Kinetics of nisin Z penetration into a monolayer of bovine heart Ptd_2Gro (curve 1) and $Ole_2GroPEtn$ (curve 2). Experimental conditions as described in Fig. 2.

Monolayer experiments. Monolayers were formed of either pure lipids or bacterial total lipid extracts on a subphase of 5 ml 10 mM Tris pH 7.4 (Demel, 1994) to an initial surface pressure (π) between 15–35 mN/m. Peptides were added through a small injection hole to the subphase which was continuously stirred. The change in surface pressure ($\Delta\pi$) after the addition of nisin to the subphase was taken as a measure of penetration of the peptide into the lipid phase.

The results shown were obtained using saturating amounts of peptide and the experiments were performed at room temperature. The surface pressure increase was measured in time until a stable surface pressure was reached. The values plotted in this study correspond to these equilibrium pressures and were obtained for final subphase concentrations of approximately 2 µg nisin/ml.

RESULTS

Monolayers of pure lipids were formed to determine possible specific lipid interactions of nisin Z, nisin A, nisin Z mutants and lacticin 481. Peptide-induced pressure changes of lipid films are interpreted as penetration of the peptide or segments of the peptide into the lipid phase. Nisin Z induced large pressure changes after interaction with anionic lipid monolayers of $Ole_2GroPGro$ and *E. coli* Ptd_2Gro (Fig. 2), with the largest changes for the latter lipid. At an initial pressure of 35 mN/m the pres-

Table 1. Antimicrobial activity of nisin and its mutants compared to pressure changes induced in $Ole_2GroPGro$ and $Ole_2GroPCho$ monolayers at initial pressures of 32 and 35 mN/m. Antimicrobial activity: +++++, >100%; ++++, 100%; +++, 50–90%; ++, 10–50%; +, 2–10%; −, <2%.

Nisin	Antimicrobial activity against *M. flavus*	Pressure change induced in			
		$Ole_2GroPGro$		$Ole_2GroPCho$	
		32 mN/m	35 mN/m	32 mN/m	35 mN/m
		mN/m			
Nisin Z	++++	8.5	6.5	1.7	0
Nisin A	++++	8.4	6.5	1.5	0
[Dhb5]Nisin Z	++	3.8	2.9	0	0
[Dha2]Nisin Z	+++++	7.5	5.6	2.3	0
[Trp17]Nisin Z	+++	9.0	6.9	0.5	0
[Trp1]Nisin Z	++	4.7	3.2	0	0
Subtilin leader nisin Z	−	0.4	0	0	0
ITPQ-nisin Z	−	3.1	1.4	0	0
[Gln17, Thr18]Nisin Z	++++	6.0	5.3	0	0
Des-(Asn20, Met21)-nisin A	+	3.3	0.9	0	0
Lactinin 481	+	6.0	4.6	3.0	1.4

sure increase is 6.5 and 9.3 mN/m for $Ole_2GroPGro$ and *E. coli* Ptd_2Gro, respectively. This brings the final film pressure very close to the collapse pressure of the lipids, which is approximately 45 mN/m. Higher pressure changes virtually cannot be measured. The pressure changes for $Pam_2GroPGro$ and bovine heart Ptd_2Gro (data not shown) were similar to those found for $Ole_2GroPGro$, indicating that there is no gross effect of fatty acyl chain composition. On the other hand, the affinity of nisin Z for zwitterionic monolayers of $Ole_2GroPCho$, $Ole_2GroPEtn$ or uncharged monolayers of $acyl_2GalGro$ or $acyl_2Gal_2Gro$ was much lower than for the anionic lipids (Fig. 2). Particularly at initial surface pressures higher than 30 mN/m, the interaction with these lipid-model membranes ceased.

The kinetics of nisin Z interaction with zwitterionic and anionic lipid layers is also different. With electrically neutral lipid layers the pressure increase is maximal after approximately 2 min (Fig. 3). However for anionic lipids the interaction could take 10–20 min to reach maximal values and showed a biphasic profile. A rapid initial pressure increase, during the first 3–5 min, is followed by a gradual change at a lower rate (Fig. 3). The results for nisin A were practically identical to those of nisin Z (Table 1). The antimicrobial activities of nisin Z and nisin A are also similar (Mulders et al., 1991).

Nisin activity is very sensitive to structural changes in the first lanthionine ring. The subtle change of dehydroalanine (Dha) at position 5 into dehydrobutyrine (Dhb) reduces its antimicrobial activity 2–10-fold (Kuipers et al., 1992). The pressure changes induced by [Dhb5]nisin Z in $Ole_2GroPGro$ monolayers were significantly reduced as well (Fig. 4, compare Fig. 2) but the lipid specificity was unaltered. At an initial surface pressure of 35 mN/m the induced changes were 3.5 and 6.5 mN/m for [Dhb5]nisin Z and nisin Z, respectively. The interactions with zwitterionic lipids were also reduced for this nisin mutant. Replacement of dehydrobutyrine by dehydroalanine at position 2 enhanced antimicrobial activity against *M. flavus* (Kuipers et al., unpublished results). The [Dha2]nisin Z mutant induced nearly equal pressure changes in $Ole_2GroPGro$ and *E. coli* Ptd_2Gro as the parent nisin Z with nearly maximal attainable effects, which means that the final pressure is close to the collapse pressure of the lipid film (Table 1).

Trp-containing mutants of nisin can be used as a tool in fluorescence spectroscopic measurements to monitor binding to and

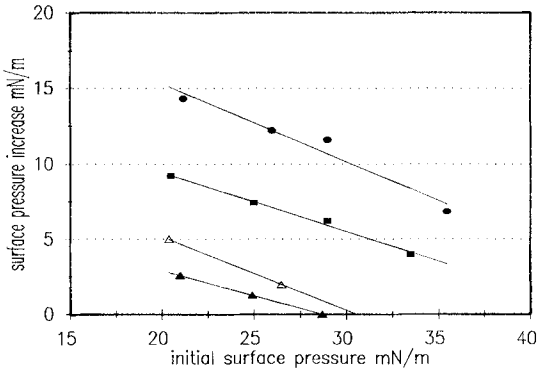

Fig. 4. Surface pressure increases after the injection of [Dhb5]nisin Z underneath monolayers of various lipids at different initial surface pressures. *E. coli* Ptd_2Gro (●); $Ole_2GroPGro$ (■), $Ole_2GroPCho$ (△); $Ole_2GroPEtn$ (▲). Experimental conditions as described in Fig. 2.

penetration of vesicle membranes (Kuipers et al., unpublished results). A Trp at position 17 did not change the interaction of nisin Z with $Ole_2GroPGro$ monolayers compared to the parent nisin Z, as was tested for [Trp17]nisin Z. However introduction of Trp at position 1, replacing Ile ([Trp1]nisin Z) showed a reduced anionic lipid interaction (Table 1) and correspondingly the antimicrobial activity is negatively affected.

Extending the N-terminus of nisin has severe effects on the antimicrobial activity of the lantibiotic. Subtilin-leader nisin Z, with a 22-amino-acid extension, identical to the leader peptide of the related lantibiotic subtilin, has little activity (Kuipers et al., 1993b) The interaction of this precursor nisin Z with $Ole_2GroPGro$ monolayers was reduced at pressures below 30 mN/m and declined rapidly to zero at a pressure of 32 mN/m (Fig. 5). The nonlinear decline in the ability to penetrate monolayers was also seen for $Ole_2GroPCho$, ceasing at a similar pressure as with $Ole_2GroPGro$. The relatively polar leader sequence contains eight charged amino acids including the N-terminus, with two positive charges in excess. The results could indicate a restriction in the ability to penetrate the hydrophobic phase of lipid membranes. Also extension of the nisin N-terminus with four amino acid residues (ITPQ-nisin Z) leads to a decrease in interaction with anionic and zwitterionic lipids.

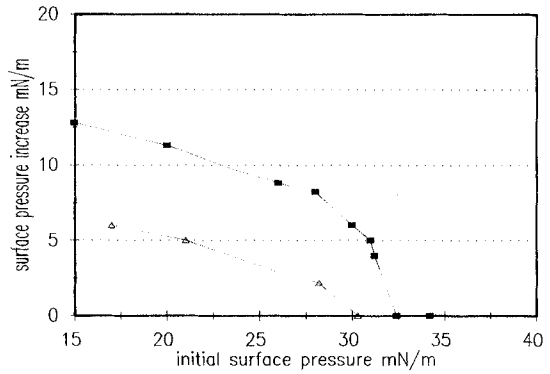

Fig. 5. Surface pressure increases after the injection of subtilin-leader nisin Z underneath monolayers of $Ole_2GroPGro$ (■) and $Ole_2GroPCho$ (△) at different initial surface pressures. Experimental conditions as described in Fig. 2.

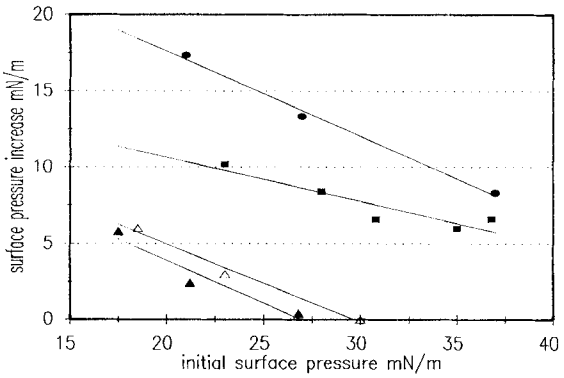

Fig. 6. Surface pressure increases after the injection of [Gln17, Thr18]nisin Z underneath monolayers of *E. coli* Ptd_2Gro (●), $Ole_2GroPGro$ (■), $Ole_2GroPCho$ (△) and $Ole_2GroPEtn$ (▲) at different initial surface pressures. Experimental conditions as described in Fig. 2.

Changes in lanthionine ring C between positions 13 and 19, also affects nisin activity (Table 1). The mutant nisin Z with Gln and Thr at position 17 and 18 [Gln17, Thr18]nisin Z has an increased activity against *M. flavus*, whereas it showed a lower activity against *B. cereus* and *S. thermophilus* (Kuipers et al., 1992). The effect of [Gln17, Thr18]nisin Z on $Ole_2GroPGro$ monolayers was a reduced slope in the $\pi_i - A\pi$ plot (Fig. 6). The pressure change was smaller at initial pressures lower than 36 mN/m but larger at initial pressures higher than 36 mN/m, relative to the parent nisin Z. The effects of [Gln17, Thr18]nisin Z with respect to *E. coli* Ptd_2Gro monolayers were similar to nisin Z.

NMR experiments indicated that nisin possesses a hinge region at positions 20–22 (Van de Ven et al., 1991). Deletion of residues 20–21 (Kuipers et al., unpublished results) could rigidify the structure and lead to a reduction in antimicrobial activity. The interaction with lipid model membranes is concomitantly drastically reduced. With $Ole_2GroPGro$ monolayers the effect of des-(Asn20, Met21)-nisin A, at initial pressures of 32 and 35 mN/m, is reduced by 60% and 85%, respectively (Table 1).

To test the specificity of interaction of another lantibiotic, the effect of lacticine 481 (Piard et al., 1992, 1993) on anionic and zwitterionic lipid monolayers was tested. Lacticine 481 which has a lower antimicrobial activity than nisin has also a lower affinity for $Ole_2GroPGro$ monolayers than nisin Z (Table 1) but interestingly its $Ole_2GroPCho$ affinity is relatively higher. The induced pressure increase by lacticine 481 in $Ole_2GroPGro$ and $Ole_2GroPCho$ monolayers at an initial pressure of 35 mN/m was 4.6 and 1.4 mN/m, respectively. This indicates a lower specificity for anionic lipids of lacticine 481 compared to nisin Z and a higher specificity for zwitterionic lipids, which correlates with the difference in antimicrobial spectrum of lacticin 481 relative to nisin.

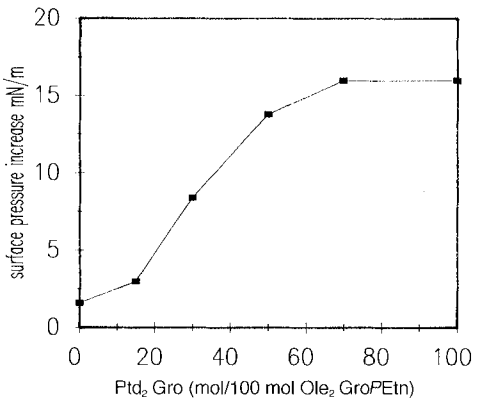

Fig. 7. Surface pressure increases after the injection of nisin Z underneath mixed monolayers of *E. coli* Ptd_2Gro and $Ole_2GroPEtn$ at an initial pressure of 27 mN/m. Experimental conditions as described in Fig. 2.

The results show that nisin interacts with high affinity with anionic lipids. The importance of ionic interactions in nisin–lipid interactions was further tested by increasing the ionic strength to 300 mM and 500 mM NaCl. The pressure increase recorded during the first 3 min after nisin Z administration to $Ole_2GroPGro$ monolayers, at initial pressures 32 mN/m, was reduced by 21% only. However, in a time course of 10–20 min there was a gradual decrease in surface pressure finally leading to a 65% reduction compared to the low-ionic-strength condition. The results indicate that high ionic strength inhibits primarily secondary interactions between nisin or oligomeric forms of nisin and anionic lipids. Although nisin has a significantly higher solubility at pH 5.0 than at pH 7.4 (Rollema et al., 1995) no significant differences were measured in monolayer experiments in the pH range 5.0–7.0 using phosphate buffers. At an initial pressure of 20 mN/m, the pressure change was slightly lower at pH 5.0 than at high pH values.

Changing the ratio of anionic and zwitterionic lipids showed that the interaction with nisin did not change linearly with increasing anionic lipid content (Fig. 7). In mixtures of $Ole_2GroPEtn$ and *E. coli* Ptd_2Gro the pressure change was not enhanced between 0–15 mol *E. coli* Ptd_2Gro/mol $Ole_2GroPEtn$ compared to pure $Ole_2GroPEtn$ but increased at higher molar percentages of *E. coli* Ptd_2Gro and became maximal at approximately 70%. This indicates that in these model membranes at least 15 mol anionic lipid/100 mol is required for efficient interaction with nisin.

The interaction of nisin with the lipids of target membranes was tested using lipid extracts of gram-positive bacteria. Lipids of two strains were used: *M. flavus* that is relatively sensitive to nisin and *L. monocytogenes* that is relatively insensitive to nisin (De Vos et al., 1993). Analysis of the lipid composition of extracts of *M. flavus* and *L. monocytogenes* revealed high concentrations of anionic lipids. For *M. flavus* the PtdGro (60%) and Ptd_2Gro (32%) amounted together to 92% of the total phospholipid extracted, whereas for *L. monocytogenes* this value is approximately 60% (PtdGro 51% and Ptd_2Gro 7%). It is known that particularly cardiolipin is not readily extracted from gram-positive bacteria and is probably complexed by the cell wall. Digestion of the cell wall by lysozyme treatment could increase

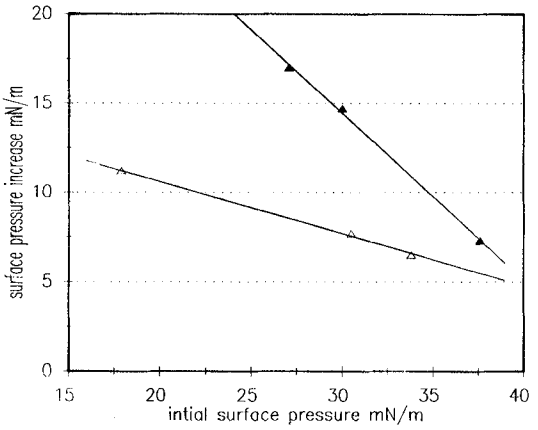

Fig. 8. Surface pressure increases after the injection of nisin Z underneath monolayers of lipid extracts from *M. flavus* (▲) and *L. monocytogenes* (△) at different initial surface pressures. Lipids were extracted in the absence of lysozyme. Experimental conditions as described in Fig. 2.

the extractability of anionic lipids. For *M. flavus*, lysozyme treatment (4 mg/ml) did not result in an increased yield of crude lipid extract from the wet cell pellet (9 mg/g wet cell pellet). *L. monocytogenes* required higher lysozyme concentrations (up to 180 mg/ml) and longer incubation times which resulted in a threefold increased yield in extracted lipid: 3 mg and 10.8 mg/g wet cell pellet were obtained in the absence and presence of lysozyme, respectively. The relative concentrations of cardiolipin increased after lysozyme treatment and, unexpectedly, also the concentration of PtdEtn. Especially *L. monocytogenes* extracts contained a phospholipid fraction which could not be positively ascribed, although the R_f values coincided with PtdIns. Extracts from both bacterial strains contained a small glycolipid fraction which stained positively with orcinol.

Monomolecular layers of lipid extracts of *M. flavus* and *L. monocytogenes* obtained without lysozyme treatment were compared for their interaction with nisin Z. Monomolecular layers of *M. flavus* lipid extracts showed high pressure increases after the addition of nisin Z (Fig. 8). The final pressures (initial surface pressure + surface pressure increase), were even higher than for pure *E. coli* Ptd_2Gro monolayers reaching 45 mN/m, the collapse pressure of most membrane lipids. The pressure changes induced by nisin Z in monolayers of *L. monocytogenes* lipid extracts were significantly lower. The differences in surface pressure reflect qualitatively the differences in nisin sensitivity of *M. flavus* and *L. monocytogenes*. Monomolecular layers of lipid extracts of *M. flavus* and *L. monocytogenes*, obtained after lysozyme treatment, both showed maximal pressure increases after nisin Z addition. Therefore possible differences could not be detected. The results were comparable to those described in Fig. 8 for *M. flavus* lipid extracts. The lipid fractions obtained after lysozyme treatment probably do not reflect the lipid fractions that potentially can interact with nisin Z in the intact bacterial membrane. Nisin mutants tested for their interaction with monolayers of *M. flavus* lipid extracts showed qualitatively the differences as demonstrated for pure $Ole_2GroPGro$ monolayers (results not shown).

DISCUSSION

The results from the studies with pure lipids demonstrate that nisin Z and nisin A have a similar high affinity for phosphatidylglycerol (GroPGro) and cardiolipin (Ptd_2Gro), the predominant anionic lipids of gram-positive bacteria. In contrast, the interaction with zwitterionic lipids is significantly lower. Particularly at surface pressures of 30–35 mN/m which are believed to be relevant for biological membranes (Demel et al., 1975) there is little or no interaction between nisin and zwitterionic lipid monolayers (Fig. 2). The molecular area of *E. coli.* Ptd_2Gro at 30 mN/m is 1.15 nm^2/molecule (Killian et al., 1994) compared to 0.716 nm^2/molecule for $Ole_2GroPGro$ (Smaal et al., 1987). This means that the surface concentration of the anionic phosphate group and therefore the charge density is highest in the case of cardiolipin. The high charge density, but also the specific charge distribution in the case of cardiolipin, is likely to determine the strong interaction with nisin. Acyl chain saturation did not have a major effect on the nisin-induced pressure change.

The mutant nisins tested showed a striking correlation between the affinity for anionic lipids in monolayers, resulting in a change in surface pressure, and their antimicrobial activity against *M. flavus*. Extension of nisin at the N-terminal Ile with 22 residues, as in subtilin leader nisin Z, inhibited penetration of the antibiotic into lipid layers at surface pressures higher than 32 mN/m (Fig. 4 and Table 1). Accordingly, the antimicrobial activity is low. A similar sharp cut-off pressure as demonstrated for subtilin-leader nisin Z in the interaction with $Ole_2GroPGro$ monolayers (Fig. 4) was seen before for the interfacial penetration of PtdCho monolayers by phospholipase C from *Bacillus cereus* where a change in surface pressure from 29 to 31 mN/m completely inhibited substrate degradation at the interface (Demel et al., 1975). Extension with only four residues as in ITPQ-nisin Z resulted already in a 3–5-fold reduction in the penetration of $Ole_2GroPGro$ monolayers and a very low antimicrobial activity (Table 1). It can be concluded that penetration of the N-terminal part of nisin into the lipid membrane is probably essential for biological activity. This view is supported by the reduced lipid penetration of nisin after replacement of the hydrophobic N-terminal Ile by a surface-seeking Trp residue (Jones and Gierasch, 1994; Table 1). Changes in the first or third lanthionine ring can also affect nisin antimicrobial activity and lipid penetration (Figs. 3 and 5 and Table 1). Dehydroalanine and dehydrobutyrine residues and intact lanthionine rings play an essential role in this respect. The presence of dehydroalanine at positions 2 and 5 has a positive effect on biological activity and $Ole_2GroPGro$ interaction. Replacement by dehydrobutyrine seems to be less favorable. By using NMR spectroscopy and distance geometry calculations, Goodman et al. (1991) found that nisin in $(C^2H_3)_2SO$ has a kinked rod-like shape with two more or less rigid parts which comprise the first three rings A, B, C on one hand and the bicycle rings D and E on the other. The two domains are connected by a flexible hinge (positions 20–22; Van de Ven et al., 1991). Blocking the hinge function of nisin by deletion of Asn and Met at positions 20 and 21 leads to reduction in anionic lipid penetration and biological activity. It is tempting to speculate that the hinge function is important for membrane insertion of the N-terminal part of nisin after initial binding to the membrane.

In view of the high affinity of nisin for anionic lipids, ionic interactions between the positively charged antibiotic and the negatively charged lipids must play a predominant role. Positively charged Lys residues are present at positions 12, 22, and 34 and at pH < 6 positively charged His at position 27 in nisin A (but not in nisin Z) and at position 31. Potentially, also the charged N-terminus can be involved in an initial ionic interaction. Elimination of Lys12 ([Pro12]nisin Z) has little effect on the antimicrobial activity of nisin, whereas the introduction of Lys at position 17 ([Lys17]nisin Z) even reduced activity (Kuipers et al., unpublished results). Therefore, it is unlikely that charged residues in the N-terminal part (residues 1–22) play an

essential role in the binding to the membrane. This leaves the possibility that the positively charged residues of the C-terminal part (residues 22–34) are involved in initial ionic interactions. In the natural variant nisin Z, His27 of nisin A is replaced by Asn without loss of activity, indicating that charge at this position is not crucial.

The kinetics of nisin interaction with anionic lipid monolayers show a second pressure increase after the initial pressure change (Fig. 2) This could indicate that there is a second further penetration step of nisin or a reorientation or oligomerisation of nisin. An effect of high ionic strength on the surface pressure is only apparent 10–20 min after the initial interaction. This could indicate that nisin dissociates again from the monolayer or that oligomeric complex formation is inhibited. It is likely that charge interactions with anionic lipids are also involved in formation of oligomeric nisin structures. From the present data it cannot be concluded yet whether the C-terminal part of nisin actually penetrates the monolayer. By using ^{1}H NMR, van den Hooven et al. (1993) determined the nisin structure in model systems of sodium dodecyl sulphate or dodecylphosphocholine. CD spectra and chemical shift data indicated that nisin adopts a different conformation than in aqueous solution with major changes in the N-terminus. The high anionic lipid specificity of lantibiotics is not found for lacticin 481 which has a relative high affinity for zwitterionic lipids (Table 1). This might be correlated to the different antimicrobial spectrum of lacticin 481 (Piard et al., 1993).

For the mutant nisins used in this study a correlation was found between the interaction with anionic lipid monolayers and the antimicrobial activity against *M. flavus*. It is likely that nisin also has affinity for anionic lipids of the target membrane of gram-positive bacteria. In these membranes phosphatidylglycerol and cardiolipin are the most abundant lipids. For monolayers of the lipid extracts of a nisin-sensitive micro-organism (*M. flavus*) a higher affinity was found than for monolayers of the lipid extract of a nisin-insensitive micro-organism (*L. monocytogenes*) at least when the extracts were obtained without lysozyme treatment. This raises the question about the fraction of anionic lipids that potentially can interact with nisin, since part of the anionic lipids might be complexed to other membrane components and divalent cations. Also the cell wall could be involved in nisin binding or act as a barrier for its membrane targeting.

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